Clin. exp. Immunol. (1978) **31**, 436–442.

Hepatocyte damage induced by lymphocytes from patients with chronic liver diseases, as detected by LDH release

E. FERNANDEZ-CRUZ,* P. ESCARTIN,† A. BOOTELLO, M. KREISLER & J. M. SEGOVIA DE ARANA *Department of Immunology, and †Department of Gastroenterology, Clinica Puerta de Hierro, Universidad Autonoma de Madrid, Madrid, Spain*

(*Received* 10 *October* 1977)

SUMMARY

We have used a cytoplasmic enzyme system in the study of the *in vitro* cytotoxic activity of human peripheral blood leucocytes against isolated liver cells in patients with chronic liver diseases. Lymphocytes from primary biliary cirrhosis and chronic active liver disease patients were shown to have an *in vitro* capacity to induce a cytolitic effect on isolated hepatocytes, as demonstrated by the enhanced release of lactate dehydrogenase (LDH), a cytoplasmic marker enzyme. No significant LDH release was seen with control lymphocytes of normal persons or with lymphocytes from patients with alcoholic cirrhosis. Our results corroborate, in a different assay system, by a simple, reproducible and different method, that lymphocyte-mediated liver cell damage '*in vitro*' occurs in both primary biliary cirrhosis and chronic active liver disease.

INTRODUCTION

The existence of an autoaggressive process in the liver has been postulated over recent years. *In vitro* studies, using a variety of methods (leucocyte migration inhibition, lymphocyte transformation and cytotoxicity tests) were carried out to provide evidence of cell mediated immune responses against liver antigens in a number of chronic liver diseases, such as chronic active liver disease (CALD) and primary biliary cirrhosis (PBC).

Cell-mediated immune reactions to liver antigens (Smith *et al.*, 1972), to liver-specific lipoprotein (Miller *et al.*, 1972; Meyer zum Buschenfelde, Knolle & Berger, 1974; Thestrup-Pedersen, Ladefoged & Andersen, 1976; Cochrane *et al.*, 1976) and to hepatitis B surface antigen (HBsAg), a marker of the hepatitis B virus (Dudley, Giustino & Sherlock, 1972; Dudley, Fox & Sherlock, 1972; Ito, Nakagawa & Okimoto, 1972; Giustino, Dudley & Sherlock, 1972; Sutnick, London & Blumberg, 1973; Eddleston & Williams, 1974; Howlett & Mcguigan, 1975), have been found in a high percentage of cases of patients with chronic active liver disease.

Studies on primary biliary cirrhosis have also shown evidence of cell-mediated immunity to a liver-specific lipoprotein (Miller *et al.*, 1972) and to a protein fraction from human bile (Eddleston *et al.*, 1973), as well as impairment of the mechanisms of delayed hypersensitivity in PBC patients (Fox *et al.*, 1969; MacSween *et al.*, 1973; Fox, Dudley & Sherlock, 1973).

Other studies, using cultured rabbit liver cells and autochthonous hepatocytes grown in tissue culture as target cells, have shown lymphocyte cytotoxicity in patients with chronic active hepatitis (CAH) and in a small number of cases of patients with PBC (Thomson *et al.*, 1974; Paronetto & Vernace, 1975). Vogten *et al.* (1976), using purified liver membrane lipoprotein coated on avian erythrocytes, has demonstrated cell-mediated cytotoxicity in PBC, CALD and in a number of miscellaneous liver diseases. Other investigations have shown the existence of cell-mediated immune responses against Chang liver cells in

Correspondence: Dr E. Fernandez-Cruz, Department of Immunopathology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037.

0099-9104/78/0300-0436 $02.00

patients with acute and chronic hepatitis (Wands *et al.*, 1975). Thomas *et al.* (1976), studying the *in vitro* capacity of lymphocytes to destroy Chang liver cells in acute and chronic liver diseases, have demonstrated that cytotoxicity was positive in acute type A and B hepatitis and chronic active hepatitis, and negative in primary biliary cirrhosis and other liver diseases.

In this work, we have used a cytoplasmic enzyme system in the study of the *in vitro* cytotoxic activity of human peripheral blood leucocytes (PBL) against isolated liver cells in patients with primary biliary cyrrhosis, chronic active liver disease, alcoholic cirrhosis and in healthy normal persons. Our method of study was based on measuring the damage to the plasma membrane of liver cells, by assessing the lactate dehydrogenase (LDH) release, a cytoplasmic marker enzyme, from isolated rat hepatocytes. The results show clearly that lymphocytes from patients with PBC are able to produce an injurious effect on the plasma membrane of isolated liver cells. In addition, we have compared these results in PBC with those found in other chronic liver diseases and in healthy adult persons.

MATERIALS AND METHODS

Patients and controls. The studies were performed in patients whose diagnoses were based on current clinical, morphological, biochemical and immunological criteria. Nine patients had PBC, eight patients had CALD, ten patients had alcoholic liver cirrhosis (AC) and twenty HBsAg-negative and healthy adult persons were used as controls. At the time of the study, none of the patients were receiving immunosuppressive drugs.

The patients with PBC comprised nine females of middle age at different morphological stages of the disease: florid duct lesions and ductular proliferation, with or without scarring. Mitochondrial antibodies accurred in nine patients and in all the cases the serum IgM level was high. Other autoimmune phenomena, such as nuclear and smooth muscle antibodies, were present in five of the patients. None had HBsAg.

Patients with CALD comprised five males and three females. HBsAg was present in the sera of six patients and nuclear and smooth muscle antibodies were present in the sera of three of the patients.

In the AC group all the patients had a previous history of heavy drinking with clinically well-defined cirrhosis, considered histologically to be cases of inactive cirrhosis. The biopsy showed the existence of alcoholic hyalin in minimal amounts in only two of the cases. The remaining patients showed minimal liver cell and piecemeal necrosis and moderate lymphocytic infiltration of the portal tracts. Patients with other possible aetiologies for their cirrhosis or with histological diagnosis of alcoholic hepatitis were excluded from the study. Antinuclear antibodies (three cases) and smooth muscle antibodies (one case), at low titres, as well as elevation of the level of the three main Ig classes were found. All of the patients were HBsAg-negative.

We used twenty healthy adults as controls. None had HBsAg.

Isolation of peripheral blood leucocytes. PBL of patients and normal volunteers were separated by Ficoll–Isopaque gradient centrifugation at 200 *g* for 40 min. The cells from the Ficoll layer were washed four times in Eagle's minimal essential medium (MEM) and the cell number was adjusted to 5, 10 and 20×10^6 viable cells per ml. Such suspensions exceeded 95% in viability (as determined by trypan blue exclusion) and consisted of 80–90% lymphocytes, 10–20% monocytes and 1–2% polymorphonuclear leucocytes.

Isolation of rat hepatocytes. Hopf, Meyer zum Buschenfelde & Freudenberg (1974), using antibody assays and immunofluorescence studies, have demonstrated complete organ, but incomplete species, specificity, with cross-reactions among rabbit, rat and human liver protein. In our work, the livers were obtained from Albino rats weighing 150 *g*, fasted for 20 hr. The technique of liver perfusion was carried out following the method described by Hopf, Meyer zum Buschenfelde & Dierich (1976), with minor modifications. The perfusion medium for the preparation of the isolated rat hepatocytes did not contain enzymes. The live fresh rat hepatocytes were prepared for each experiment and were then filtered through nylon wool and centrifuged four times at 900 rev/min (56 *g*) for 10 min in a culture medium containing 0·154 M KCl, 0·154 M KH_2PO_4, 0·154 M $MgSO_4{\cdot}7H_2O$, 0·154 M NaCl and 0·127 M CO_3HNa (pH 7·4), supplemented with bovine serum albumin (2·5% (Bovine albumin powder, Fraction V, Sigma Chemical Co., U.S.A.) and exposed to 95% oxygen and 5% CO_2. The viability of the hepatocyte preparation (over 95%) was examined by the trypan blue (0·5%) and erythrosin (1%) techniques with phase contrast microscopy. The assays with fresh hepatocytes were always completed within 1 and 4 hr after isolation.

Measurement of LDH. Plasma membrane hepatocyte damage was determined by the release of LDH, a cytoplasmic marker enzyme that has been shown to be a sensitive index for cell lysis (Davies *et al.*, 1973; Ranadive *et al.*, 1973; Segal & Levi, 1975; Sajnani, Ranavide & Movat, 1976).

In our enzyme assay, PBL ($5–20\times 10^6$ viable cells per ml) were mixed with 10^6 viable rat hepatocytes in 1 ml of the culture medium supplemented with 0·25% bovine serum albumin for 30 min at 37°C in a humid atmosphere of 5% CO_2 and air. After incubation, the cells were removed by gentle centrifugation and an aliquot of the supernatant of the mixture was transferred to a spectrophotometer cuvette containing 3 ml assay medium (0·05 M phosphate buffer, pH 7·4, $3{\cdot}1\times 10^{-4}$ M pyruvate and $1{\cdot}3\times 10^{-4}$ M β-NADH) (β-Nicotinamide-adenine-dinucleotide reduziert, Boehringer–Mannheim) and placed in a spectrophotometer (DMR-21, Zeiss). The release of LDH from hepatocytes was then immediately assayed by

determining the rate of oxidation of the reduced nicotinamide adenine dinucleotide at 340 nm (Bergmeyer, Berut & Hess, 1963). The results were expressed in milliunits of LDH per min (mu/min), defining 1 unit as the amount of LDH capable under our conditions of oxidizing 1 mol of NADH per min. The reproducibility of the assay in duplicates never exceeded 6%.

Differences among values were calculated for significance by the unpaired Students *t*-test.

RESULTS

In our experimental system, membrane hepatocyte damage was estimated by the number of mu of LDH released from hepatocytes (10^6 viable cells) following incubation with increasing numbers of PBC ($5–20 \times 10^6$ cells per ml) from patients with PBC, CALD, AC and from healthy controls.

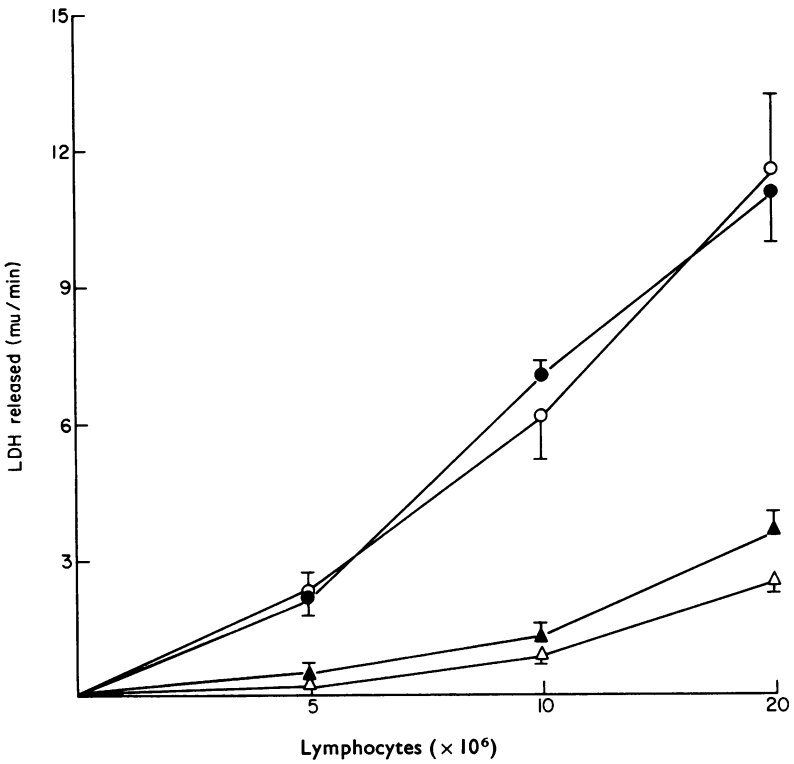

FIG. 1. Measurement of the release of the cytoplasmic marker LDH from rat hepatocytes exposed to various concentrations of lymphocytes from patients with primary biliary cirrhosis (nine cases), chronic active hepatitis (eight cases), alcoholic cirrhosis (ten cases) and normal control donors (twenty cases). Each point of the graph represents the mean ± s.e.m. of mu of LDH released from rat liver cells, using a constant target cell concentration (10^6 viable cells per ml). Incubation time 30 min at 37°C. (○) Lymphocytes from primary biliary cirrhosis; (●) lymphocytes from chronic active hepatitis; (▲) lymphocytes from alcoholic cirrhosis; (△) lymphocytes from control donors.

In the experimental procedures, control hepatocytes (10^6 per ml) incubated in the absence of lymphocytes did not release a significant amount of the cytoplasmic marker LDH (<0·3 mu/min LDH per 10^6 hepatocytes). The destruction of the liver cells by sonication allowed a total LDH release of 20 mu per 10^6 cells.

Control and patient PBL, in the absence of hepatocytes, at a concentration of 10^6 cells per ml showed an insignificant LDH release (mean ± standard error of the mean) of 0·11 ± 0·02 mu/min LDH per 10^6 lymphocytes. However, when increasing numbers of PBL were used, the release of LDH varied from

0·5±0·16 mu/min LDH (5×10^6 cells per ml) to 2·57±0·20 mu/min LDH (20×10^6 cells per ml) under various conditions.

Hepatocytes incubated in the presence of normal human PBL showed an LDH release whose values did not increase above a low base-line level, ranging from 0·21±0·09 mu/min LDH (ratio of lymphocytes: hepatocytes of 5:1) to 2·61±0·29 mu/min LDH (ratio of 20:1), indicating a lack of cytolytic effect. The trypan blue and erythrosyn dye tests also showed that there was no significant increase in the number of cells taking up the dye in the controls.

However, as it is shown in Fig. 1, the PBL from patients with primary biliary cirrhosis were cytotoxic

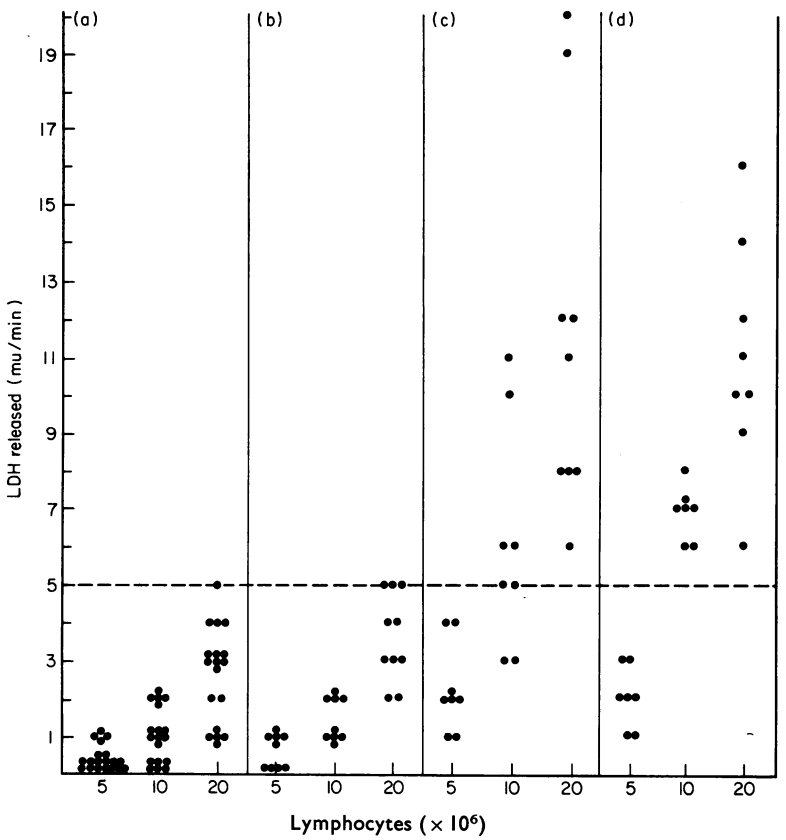

FIG. 2. Distribution of results (mu of LDH released per min from 10^6 isolated hepatocytes) for the three groups of patients in comparison with normal controls. The dashed line represents the limit of the highest values, at a lymphocyte:hepatocyte ratio of 20:1, obtained in ten alcoholic cirrhosis patients and in twenty healthy laboratory controls. (a) Controls (n = 20); (b) alcoholic cirrhosis (n = 10); (c) primary biliary cirrhosis (n = 9); (d) active chronic hepatitis (n = 8).

to rat liver hepatocytes, showing a significant LDH release when tested at a lymphocyte:target cell ratio which varied from 5:1 (2·25±0·41 mu/min LDH) to 20:1 (11·55±1·65 mu/min LDH). The enhanced LDH released from hepatocytes by increasing numbers of PBL in patients with PBC was significantly different from the group of normal subjects ($P<0·001$).

The LDH release from hepatocytes was also substantially increased by PBL from patients with CALD, as is shown in Fig. 1. At a lymphocyte:liver cell ratio of 20:1, the mean LDH released was 11±1·08 mu/min LDH as compared to 2·61±0·29 mu/min LDH in normal control individuals ($P<$ 0·001). There was no significant difference between the capacity of PBL from patients with PBC and CALD in inducing the release of LDH from hepatocytes (P = 0·788). However, there was a significant difference between the lymphocytes from patients with PBC and CALD and the lymphocytes from the

group of patients with AC, which showed a significantly lower capacity to induce the release of LDH from the hepatocytes. The values varied from the 5:1 ratio (mean: 0·5±0·18 mu/min LDH) to the 20:1 ratio (mean: 3·6±0·37 mu min LDH) ($P<0·001$). Furthermore, in the AC group, in contrast with the PBC and CALD groups, only at the PBL:target cell ratio of 20:1 was the mean LDH released from hepatocytes slightly different from the group of normal controls ($P = 0·05$).

PBC and CALD showed a positive relationship between the increase in release of the cytoplasmic marker LDH and the increase in the number of liver cells which failed to exclude trypan blue, as observed by phase-contrast microscopy (not shown). However, in AC, the LDH released did not differ significantly from control values and 95–99% of the liver cells excluded the supravital dye.

In Fig. 2 the distribution of individual values of each patient in each group is shown. On the graph we have drawn a broken line (for the 20:1 lymphocyte:liver cell ratio) at 5 mu/min LDH release, which delineates a region where the highest values found in the group of patients with AC and healthy contols approach the lowest values found in the group of patients of PBC and CALD. This distribution would be similar to the one that could be obtained by calculating the confidence interval in the control ($2·61±2·11×1·24 = 2·61±2·62 = -0·01–5·23$). It is also shown that among the group of patients with AC and the healthy controls the data were generally grouped closer together, whereas among the groups of PBC and CALD patients the data showed a more dispersed distribution. The dispersion in the data distribution in PBC and CALD could be related to the varied stages of the disease and/or to the duration of the disease and to its treatment, in accordance with Geubel *et al.* (1976). However, since our observations are based on few cases we prefer not to draw any conclusions at this time.

DISCUSSION

The enhanced release of the cytoplasmic marker LDH, from the freshly isolated hepatocytes exposed to PBL from patients with PBC and CALD, was indicative of damage to the plasma membrane. This cytolytic effect has proved to be a process dependent upon lymphocyte concentration. However, in the experiments with PBL from patients with AC and from healthy controls, the release of LDH was low in all cases, suggesting that specific cell lysis had not occurred.

Our results in the AC group are in agreement with the recent data of Cochrane *et al.* (1977), which demonstrated lymphocyte cytotoxicity for isolated hepatocytes in acute alcoholic hepatitis but not in other forms of alcoholic liver disease.

Our experiments in CALD patients confirmed that cytotoxicity occurs in HBsAg-positive and -negative cases, as reported by other authors using different methods (Geubel *et al.*, 1976; Cochrane *et al.*, 1976). However, the existence of some contradictory data on lymphocyte cytotoxicity in PBC, when liver cells grown *in vitro* are used as target cells, could have as an explanation the possible appearance of changes in the antigenicity of hepatic cells during cell replication in tissue culture (Paronetto & Vernace, 1975).

Our group of patients with PBC were in the morphological stages of florid duct lesions and ductular proliferation, and all lymphocytes from nine patients (100%) exhibited significant cytotoxicity. These data corroborate the results of Geubel *et al.* (1976) showing that, in PBC, the degree of cytotoxicity was significantly higher in patients with either florid duct lesions or ductular proliferation, rather than in those patients with the late features of scarring or cirrhosis.

Recognition and binding of target antigens by committed lymphocytes in the membrane of the cells may trigger cell-mediated cytotoxic reactions in some systems (Perlmann & Holm, 1969; MacLennan, Loewi & Howard, 1969). In CALD, a liver membrane-specific lipoprotein (LSP) (Meyer zum Buschenfeld & Miescher, 1972; Hopf *et al.*, 1974; McFarlane *et al.*, 1977) has been implicated as mainly responsible for *in vitro* lymphocyte cytotoxicity.

Vogten *et al.* (1976) has reported that normal liver cell lipoprotein(s) may be the antigen implicated in the cell-mediated cytotoxic reaction in a number of liver diseases (CALD, PBC and miscellaneous liver diseases).

In previous studies on PBC patients, we have shown the ability of lymphocytes to interact with the

surface membrane of cytoplasmic structures of the liver cells (mitochondria), with the subsequent production of mitochondrial membrane damage. This phenomenon might be a consequence of sensitization *in vivo* of the PBC patients' lymphocytes by the mitochondrial antigen(s) (Bootello *et al.*, 1976). We have also reported that mitochondrial antigens were capable of priming rabbit lymphocytes *in vivo*, and that these sensitized lymphocytes produced a total inhibition of mitochondrial respiration (Bootello *et all*, 1975).

In the present study, we have demonstrated that PBL of PBC patients may interact in a similar way, with the surface membrane of whole liver cells. This phenomenon would suggest lymphocyte reactivity to a membrane component with common or similar antigenic determinants to both cellular and subcellular structures.

However, the antigen(s) implicated in the cell-mediated cytotoxic reaction in PBC (liver cell lipoprotein(s), bile antigens and mitochondrial antigen(s)) has not been clearly defined.

At present, the nature of the effector cell mediating cytotoxicity in our assay system is unknown. The utilization of highly purified cell populations is needed for further characterization of the cell responsible for this hepatocytotoxicity. Several authors, by assesing lymphocyte-mediated cytotoxicity in acute and chronic liver diseases in a different assay system and using a variety of target cells, have shown several effector cell types: a T cell (Sheik *et al.*, 1976) and a cell which has the characteristics of a K cell (Cochrane *et al.*, 1976; Thomas *et al.*, 1976). However, up to now, the nature of the effector cells mediating antibody-dependent cell-mediated cytotoxicity appears to be dependent on the model system used (McDonald *et al.*, 1976).

Our findings have shown that the LDH assay offers a new, simple and reproducible technique for the study of the lymphocyte effector functions which might be of relevance to the mechanisms of cell damage in autoimmune chronic liver diseases. In addition, we have corroborated that *in vitro* lymphocyte-mediated liver cell damage occurs in both PBC and CALD. Further work on the identification of the membrane antigen(s) involved in the mechanisms of cytotoxicity in PBC, CALD and other chronic liver diseases is necessary. It is important to find out if this phenomenon, presumably not disease-specific (as suggested by Geubel *et al.*, 1976), might reflect the sensitization *in vivo* of the PBC and CALD patients' lymphocytes by a similar liver membrane antigenic component(s).

Lastly, the results do not infer that similar mechanisms are involved in the two processes, because indistinguishable effects have been observed in lymphocyte-mediated liver cell damage. However, the two liver syndromes can be seen to resemble each other in certain particulars: both are associated with the same range of tissue antibodies and cell-mediated immune responses, suggesting a strong immunological overlap between these two diseases. These phenomena would suggest the existence of a related autoimmunizing process associated with progressive liver injury in those postulated autoimmune liver diseases (Doniach & Walker, 1969; Doniach, 1970).

We gratefully acknowledge the valuable advice and helpful criticism of the manuscript by Professor Deborah Doniach and sincerely thank Dr Mario Gosalvez, of the Department of Experimental Biochemistry, for expert technical advice and Dr Rodes, Dr Hernandez-Gulo and Dr Pajares for their valuable interest and help. We are indebted to Dr Carmen Gutierrez for help with the immunofluorescence studies and to Ms C. Pascau, F. Blanco, L. Huerta and P. Garcia Angulo for skilled technical assistance.

This work was supported by INP grants No CPH 1231376 and 1216575.

REFERENCES

BERGMEYER, H.U., BERUT, G. & HESS, B. (1963) Lactic dehydrogenase. *Bergmeyer's Methods of Enzymatic Analysis*, p. 736. Academic Press, New York.

BOOTELLO, A., FERNANDEZ-CRUZ, E., ESCARTIN, P., BLANCO, M., GOSALVEZ, M. & SEGOVIA DE ARANA, J.M. (1976) Injurious effect on rat liver mitochondria by lymphocytes from patients with primary biliary cirrhosis. *Clin. exp. Immunol.* 24, 374.

BOOTELLO, A., FERNANDEZ-CRUZ, E., KREISLER, M., ARNAIZ, A., JIMENEZ, L., SEGOVIA DE ARANA, J.M., GOSALVEL, M. & BLANCO, M. (1975) Impairment of mitochondrial respiratory control by activated lymphocytes. *Int. Arch. Allergy appl. Immunol.* 48, 564.

COCHRANE, A.M.G., MOUSSOUROS, A., PORTMANN, B., MCFARLANE, I.G., THOMSON, A.D., EDDLESFON, A.L.W. F. & WILLIAMS, R. (1977) Lymphocyte cytotoxicity for isolated hepatocytes in alcoholic liver disease. *Gastroenterology*, 72, 918.

COCHRANE, A.M.G., MOUSSOUROS, A., THOMSON, A.D., EDDLESTON, A.L.W.F. & WILLIAM, R. (1976) Antibody-

dependent cell-mediated (K cell) cytotoxicity against isolated hepatocytes in chronic active hepatitis. *Lancet*, i, 441.

DAVIES, P., FOX, R.I., POLYZONIS, M., ALLISON, A.C. & HASWELL, A.D. (1973) The inhibition of phagocytosis and facilitation of exocytosis in rabbit polymorphonuclear leucocytes by cytochalasin B. *Lab. Invest.* **28**, 16.

DONIACH, D. (1970) The concept of an 'autoallergic' hepatitis *Proc. R. Soc. Med.* **63**, 527.

DONIACH, D. & WALKER, I.G. (1969) A unified concept of autoimmune hepatitis. *Lancet*, i, 813.

DUDLEY, F.J., FOX, R.A. & SHERLOCK, S. (1972) Cellular immunity and hepatitis-associated australia antigen liver disease. *Lancet*, i, 723.

DUDLEY, F.J., GIUSTINO, V. & SHERLOCK, S. (1972) Cell-mediated immunity in patients positive for hepatitis-associated antigen. *Brit. med. J.* iv, 754.

EDDLESTON, A.L.W.F., MCFARLANE, I.G., MITCHELL, C.G., REED, W.D., & WILLIAMS, R. (1973) Cell-mediated immune response in primary biliary cirrhosis to a protein fraction from human bile. *Brit. med. J.* iv, 274.

EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Inadequate antibody response to HBs Ag or suppressor T-cell defect in development of active chronic hepatitis. *Lancet*, ii, 1543.

FOX, R.A., DUDLEY, F.J. & SHERLOCK, S. (1973) The primary immune response to haemocyanin in patients with primary biliary cirrhosis. *Clin. exp. Immunol.* **14**, 473.

FOX, R.A., JAMES, D.G., SCHEUER, P.J., SHARMA, O. & SHERLOCK, S. (1969) Impaired delayed hypersensitivity in primary biliary cirrhosis. *Lancet*, i, 959.

GEUBEL, A.P., KELLER, R.H., SUMMERSKILL, W.H.J., DICKSON, E.R., TOMASI, T.B. & SHORTER, R.G. (1976) Lymphocyte cytotoxicity and inhibition studied with autologous liver cells: observations in chronic active liver disease and the primary biliary cirrhosis syndrome. *Gastroenterology*, **71**, 450.

GIUSTINO, V., DUDLEY, F.J. & SHERLOCK, S. (1972) Thymus dependent lymphocyte function in patients with hepatitis-associated antigen. *Lancet*, ii, 850.

HOPF, U., MEYER ZUM BUSCHENFELDE, K.H. & DIERICH, M.P. (1976) Demonstration of binding sites for IgG Fc and the third complement (C3) on isolated hepatocytes. *J. Immunol.* **117**, 639.

HOPF, U., MEYER ZUM BUSCHENFELDE, K.H. & FREUDENBERG, J. (1974) Liver-specific antigens of different species. II. Localization of a membrane antigen at cell surface of isolated hepatocytes. *Clin. exp. Immunol.* **16**, 117.

HOWLETT, S.Å. & MCGUIGAN, J.E. (1975) Inhibition of macrophage migration in response to hepatitis B surface antigen (HBsAg). *Gastroenterology*, **69**, 960.

ITO, K., NAKAGAWA, J. & OKIMOTO, Y. (1972) Chronic hepatitis: migration inhibition of leucocytes in presence of Australian antigen. *N. Engl. J. Med.* **286**, 1005.

MACLENNAN, I.C.M., LOEWI, G. & HOWARD, A. (1969) A human serum immunoglobulin with specificity for certain homologous target cells which induces target cell damage by normal human lymphocytes. *Immunology*, **17**, 897.

MACSWEEN, R.N.M., GALBRAITH, J., THOMAS, M.A., WATKINSON, G. & LUDLAN, G.B. (1973) Phytohaemagglutinin-induced lymphocyte transformation and toxoplasma Gondii antibody studies in primary biliary cirrhosis. *Clin. exp. Immunol.* **15**, 35.

MCDONALD, H.R., BONNARD, G.D., SORDAT, B. & ZAWODNIK, S.A. (1975) Antibody-dependent cell-mediated cytotoxicity: heterogeneity of effector cells in human peripheral blood. *Scand. J. Immunol.* **4**, 487.

MCFARLANE, I.G., WOJCICKA, D.M., ZUCKER, G.M., EDDELSTON, A.L.W.F. & WILLIAMS, R. (1977) Purification and characterization of human liver-specific membrane lipoprotein (LSP). *Clin. exp. Immunol.* **27**, 381.

MEYER ZUM BUSCHENFELDE, K.H., KNOLLE, J. & BERGER, J. (1974) Zellulare Immunoreaktionen gegenuber homologen lebersperifischen antigenen bei chronischen leberenzundungen. *Klin. Wschr.* **52**, 246.

MEYER ZUM BUSCHENFELDE, K.H. & MIESCHER, P.A. (1972) Liver-specific antigens: purification and characterization. *Clin. exp. Immunol.* **10**, 89.

MILLER, J., SMITH, M.G.M., MITCHELL, C.G., REED, W.D., EDDELSTON, A.L.W.F. & WILLIAMS, R. (1972) Cell-mediated immunity to a liver specific antigen (human), in patients with active chronic hepatitis and primary biliary cirrhosis. *Lancet*, ii, 296.

PARONETTO, F. & VERNACE, S. (1975) Immunological studies in patients with chronic active hepatitis. *Clin. exp. Immunol.* **19**, 99.

PERLMANN, P. & HOLM, G. (1969) Cytotoxic effects of lymphoid cells in vitro. *Adv. Immunol.* **11**, 117.

RANADIVE, N.S., SAJNANI, A.N., ALIMURKA, K. & MOVAT, H.Z. (1973) Release of basic proteins and lysosomal enzymes from neutrophil leukocytes of the rabbit. *Int. Arch. Allergy appl. Immunol.* **45**, 880.

SAJNANI, A.N., RANAVIDE, N.S. & MOVAT, H.Z. (1976) Redistribution of immunoglobulin receptors on human neutrophils and its relationship to the release of lysosomal enzymes. *Lab. Invest.* **35**, 143.

SEGAL, A.W. & LEVI, A.J. (1975) Cell damage and dye reduction in the quantitative nitroblue tetrazolium (NBT) test. *Clin. exp. Immunol.* **19**, 309.

SHEIKH, N.E.L., OSMAN, C.G., CULLENS, H., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1976) T-lymphocyte mediated cytotoxicity in HBsAg-positive acute and chronic liver disease: development of a new assay *Digestion*, **14**, Abst, 549.

SMITH, M.G.M., GOLDING, P.L., EDDLESTON, A.L.W.F., MITCHELL, C.G. & KEMP, A. (1972) Cell-mediated immune responses in chronic liver diseases. *Brit. med. J.* i, 527.

SUTNICK, A.I., LONDON, W.T. & BLUMBERG, B.S. (1973) Lymphocyte transformation and persisten Australia antigen. *Lancet*, i, 1124.

THESTRUP-PEDERSEN, K., LADEFOGED, K. & ANDERSEN, P. (1976) Lymphocyte transformation test with liver-specific protein and phytohaemagglutinin in patients with liver disease. *Clin. exp. Immunol.* **24**, 1.

THOMAS, H.C., JACQUES, E., VILLIERS, D. DE & SHERLOCK, S. (1976) Lymphocyte mediated cytotoxicity for Chang liver cells in acute and chronic liver disease. *Gut*, **17**, Abst, 389.

THOMSON, A.D., COCHRANE, M.A.G., MCFARLANE, I.G., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. *Nature (Lond.)*, **252**, 721.

WANDS, J.R., PERROTTO, J.L., ALPERT, E., ISSELBACHER, K.J. (1975) Cell-mediated immunity in acute and chronic hepatitis. *J. clin. Invest.* **55**, 921.

VOGTEN, A.J.M., SHORTER, R.G., SUMMERSKILL, W.H.J., KELLER, R.H. & TOMASI, T.B. (1976) Cell-mediated cytotoxicity against human liver, lipoprotein in liver disease. *Gastroenterology*, **71**, Abst, 5.